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Intra-specific DNA polymorphism in pineapple (*Ananas comosus* (L.) Merr.) assessed by AFLP markers

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Abstract

Pineapple (*Ananas comosus* (L.) Merr.) cultivars, often derived from somatic mutations, are propagated vegetatively. It has been suggested by isozyme data that there is little genetic variation among Smooth Cayenne cultivars. A thorough investigation of the genetic variation within the cultivated species *Ananas comosus*, particularly among commercial cultivars, will provide critical information needed for crop improvement and cultivar protection. One-hundred and forty-eight accessions of *A. comosus* and 14 accessions of related species were evaluated with AFLP markers. The average genetic similarity of *A. comosus* was 0.735 ranging from 0.549 to 0.972, suggesting a high degree of genetic variation within this species. With AFLP markers, discrete DNA fingerprints were detected for each commercial cultivar, breeding line, and intra-specific hybrid. Self-incompatibility, high levels of somatic mutation, and intra-specific hybridization may account for this high degree of variation. However, major cultivar groups of pineapple, such as Cayenne, Spanish, and Queen, could not be distinctively separated. These cultivar groups are based on morphological similarity, and the similar appearance can be caused by a few mutations that occurred on different genetic background. Our results suggest that there is abundant genetic variation within existing pineapple germplasm for selection, and discrete DNA fingerprinting patterns for commercial cultivars can be detected for cultivar protection. The genetic diversity and relationships of four *Ananas* species are also discussed.

Introduction

The genus *Ananas* from the family Bromeliaceae are herbaceous perennials with syncarps bearing foliacious bracts at maturity (Collins 1960). Eight species within *Ananas* have been described according to their differences in syncarp size, floral bracts, and presence of leaf spines (Duval and d'Eeckenbrugge 1993). The classification of *Ananas* species according to morphological traits has been inconsistent because these characters are affected by environment (Collins 1960; Aradhya et al. 1994). Moreover, DNA variations within *Ananas* appear to be continuous and *Ananas* species were not separated clearly when restriction fragment length polymorphism (RFLP) markers were used to analyze 301 *Ananas* accessions (Duval et al. 2001). Wild species *A. ananassoides* (Baker) L.B. Smith and *A. parguazensis* L.A. Camargo and L.B. Smith were shown to have higher diversity rates than the cultivated *A. comosus* (L.) Merr. and *A. bracteatus* (Lindley) Schultes f. The vegetative

propagation of cultivated species is believed to be responsible for the lower rates of variation. It was suggested that even the low levels of sexual reproduction in wild species may be significant in increasing their levels of genetic diversity (Duval et al. 2001).

In the cultivated species *Ananas comosus*, cultivars are often divided into different groups based on leaf and fruit characters. The following cultivar groups are documented: Cayenne, Spanish, Queen, Maipure, Abacaki, Mordilona, and Pernambuco (Samuels 1970; DeWald et al. 1988; Duval and d'Eeckenbrugge 1993; Noyer et al. 1995). Duval and d'Eeckenbrugge (1993) extensively analyzed five cultivar groups with 18 qualitative and 27 quantitative variables including vegetative, floral, and fruit characters. The Cayenne, Queen, and Pernambuco groups were separated distinctively and appeared to be homogenous, while two subgroups were found in Mordilona and Spanish. These cultivar groups appear to be distinct in morphology, but their genetic base is not clear. It should be noted that some cultivars do not fit in any group.

Previous isozyme studies to characterize the *Ananas* germplasm produced only a limited number of markers, up to 31 alleles at 10 loci (DeWald et al. 1988, 1992; Aradhya et al. 1994). DeWald et al. (1988) reported unique identification of 15 of the 27 cultivars examined using two peroxidase and three phosphoglucomutase loci. Aradhya et al. (1994) used six isozyme systems involving seven putative loci to study 161 pineapple accessions. Multivariate analysis of isozyme variation data did not support the *A. comosus* group classifications based on phenotypic traits. They concluded that five genetically diverse groups exist within the cultivated species *A. comosus*. However, 30 of the 31 Hawaiian 'Cayenne' accessions examined were represented by only three similar zymotypes.

Previous studies using DNA markers provided limited information on genetic variation among pineapple cultivars. Noyer (1991) reported low cytoplasmic genetic diversity among 75 pineapple accessions using seven RFLP markers. Only two cytoplasmic groups could be defined. This group also studied rDNA variability in *Ananas* genus using one wheat rDNA probe pTA71, and six groups were identified by this rDNA probe, roughly corresponding to different species within the genus (Noyer et al. 1995). Since only one Smooth Cayenne cultivar was included in their studies, these reports provided no information regarding the DNA variation within this cultivar group. Duval et al. (2001) reported DNA polymorphisms among cultivated varieties using 18 pineapple genomic DNA probes. The major cultivar groups Cayenne, Queen, and Pérola were separated into different clusters.

The PCR-based technique for the detection of restriction fragments, or amplified fragment length polymorphism (AFLP) analysis (Vos et al. 1995), was shown to be more effective than RFLP and microsatellites for the assessment of genome diversity (Breyne et al. 1999; Han et al. 2000; Robinson and Harris 1999). Advantages of the AFLP technique include the generation of large amounts of polymorphism data without having any prior sequence information, and a high multiplex ratio for the distributed analysis of the entire genome (Robinson and Harris 1999). The objectives of this project are as follows: (i) to access the genomic variability of pineapple cultivars, particularly the Cayenne group; (ii) to determine the genetic relationships of *Ananas* species; (iii) to enhance germplasm management through DNA fingerprinting of the current collection.

Materials and methods

Plant material

Young vegetative leaf material (the 4th–7th youngest leaves) from 144 *Ananas* accessions was collected from the National Clonal Germplasm Repository at Hilo, Hawaii. Eighteen additional accessions were collected from four private Hawaii-based pineapple companies. We limited our study to this collection of all available pineapple germplasm in Hawaii, even though we recognize that some *Ananas* species and pineapple variety groups had fewer accessions and were not equally represented (Tables 1 and 2). In total 162 accessions were obtained and quickly frozen at −80 °C upon arrival to the laboratory. The leaf tissue was lyophilized (FTS Systems, Inc; NY) from 72 to 120 h depending on the succulence of the material. The dried material was ground to fine powder using a Udy Sample Mill (Udy Corp, CO).

Table 1. List of Ananas accessions, species, and identity.

Accession	Identity	Accession	Identity	Accession	Identity
Set 1:					
A. comosus (L.) Merr.		*A. comosus* (L.) Merr. (cont.)		*A. comosus* (L.) Merr. (cont.)	
Cayenne		*Other*		*Other (cont.)*	
Cayenne01	F153	Ac01	Congo	Ac32	UnknownForeign Variety
Cayenne02	D10	Ac02	Spanish Samoa	Ac33	Rio Kanari
Cayenne03	F200	Ac03	Santa Marta No. 1	Ac34	CB 18
Cayenne04	Cayenne Lot 520 WBC	Ac04	Sam Clarke	Ac35	CB 30
Cayenne05	Cayenne Lanai	Ac05	Philippine Red	Ac36	CB 32
Cayenne06	Cayenne M 263 Narrow Lf	Ac06	Wild Kailua	Ac37	CB 36
Cayenne07	Cayenne Hilo	Ac07	Dacca	Ac38	Jandaira
Cayenne08	Columbia Variety No. 1	Ac08	Sylhet Jaldubi	Ac39	Rezende
Cayenne09	Sarawak	Ac09	Black Antigua	Ac40	Trinidad
Cayenne10	Sugar Loaf	Ac10	Ananas Kendal	*A. ananassoides* (Baker) L.B. Smith	
Cayenne11	Cayenne 573	Ac11	Monte Liro	Aa01	CB 6
Cayenne12	Cayenne 1069	Ac12	Amalsad	Aa03	CB 15
Cayenne13	Cayenne 7898 QC	Ac13	Cowboy	Aa04	CB 19
Cayenne14	Cayenne 45 NO. 5 4 N	Ac14	Criolla	Aa05	CB 61
Queen		Ac15	Pho Lang Tuang	*A. bracteatus* (Lindley) Schultes f.	
Queen01	Pernambuco	Ac16	Saigon Red	Ab01	F 101
Queen02	Natal	Ac17	MO	Ab02	CB 5
Queen03	Abacaxi	Ac18	MOE	Ab03	CB 11
Queen04	MacGregor	Ac19	NEP	Ab04	CB 20_rudis
Spanish		Ac20	Sugar Loaf	Ab05	CB 23
Spanish01	Ruby	Ac21	Canterra	Ab06	Albus
Spanish02	Bermuda	Ac22	Mexican Criolla	Ab07	CB 73_albus
Spanish03	Mauritus	Ac23	Pina Criolla	*A. erectifolius* L.B. Smith	
Spanish04	Spanish Guatemala	Ac24	Bogota	Ae01	CB 2
Intra-specific Hybrid		Ac25	Ananas De Vaupes	Ae02	CB 9
Hybrid01	Philippine hybridAcXAc	Ac26	PapuriVaupesColombia	*A. nanus* (L.B. Smith) L.B. Smith	
Hybrid02	Wild Brazil X Lot 520	Ac27	British Samoa P1	An01	CB 71
Hybrid03	F1 Hybrid Campinas	Ac28	British Samoa P5	*Interspecific Hybrid*	
Hybrid04	581184:C8SG4R2Q1P1	Ac29	Apaporis	ISH01	F1 Aa X AcRondon
		Ac30	Apaporis P1	ISH02	59656:C9P2SG2Q2Aa1
		Ac31	CultivadeParanaPichuna	ISH03	612223:C9Q2SG1R1P1Aa1
Set 2:					
A. ananassoides (Baker) L.B. Smith		*A. comosus* (L.) Merr. (cont.)		*A. comosus* (L.) Merr. (cont.)	
Aa03	CB 15	*Other (cont.)*		*Other (cont.)*	
A. bracteatus (Lindley) Schultes f.		Ac41	ACC. 253	Ac70	63-759
Ab02	CB 5	Ac42	Los Banos	Ac71	57-503
A. comosus (L.) Merr.		Ac43	Amarillo	Ac72	58-1184
Cayenne		Ac44	UHI	Ac73	53-116
Cayenne01	Cayenne F153	Ac45	Red Spanish	Ac74	58-474
Cayenne15	Cayenne #31 4 N	Ac46	Taboga	Ac75	N91-06
Cayenne16	Cayenne M 4W	Ac47	Jamaica Sugar	Ac76	32424
Cayenne17	Cayenne M 24	Ac48	Smooth Anpi	Ac77	75-50
Cayenne18	Cayenne M 61 Low Bloom	Ac49	Kohi	Ac78	Singapore
Cayenne19	Cayenne M 63 Plus Bloom	Ac50	Spiny Anpi	Ac79	Miami
Cayenne20	Cayenne M 91 Big Eye	Ac51	Philippine Green	Ac80	Ac0102
Cayenne21	Cayenne Seedy No. 24	Ac52	Klajatan	Ac81	Ac0103
Cayenne22	Cayenne Flowering Beauty	Ac53	Ananas Merah	Ac82	Ac0105
Cayenne23	Cayenne M 109-5	Ac54	Cheese Pine	Ac83	Ac0106
Cayenne24	Cayenne Paper Leaf	Ac55	Kumta	Ac84	Ac0107
Cayenne25	Cayenne M 262	Ac56	Morada	Ac85	Lao Kay
Cayenne26	Cayenne Bottleneck	Ac57	Pakse	Ac86	Queen Australia

Continued on next page

Table 1. Continued.

Accession	Identity	Accession	Identity	Accession	Identity
Cayenne27	Cayenne M 226 Nubby	Ac58	DO	Ac87	Red Spanish
Cayenne28	Cayenne CPC Big Eye	Ac59	DEN	Ac88	Pakse
Cayenne29	Cayenne M 267 Dry Sweet	Ac60	Pina De Castilla	Ac89	Ruby
Cayenne30	Kew	Ac61	Manzana	Ac90	Pernambuco
Cayenne31	White Flesh Cayenne	Ac62	CB 24	Ac91	Ac0301
Cayenne32	Cayenne John Teves	Ac63	CB 33	Ac92	Ac0302
Queen		Ac64	CB 38	Ac93	Ac0303
Queen05	Antigua	Ac65	CB 46	Ac94	Ac0304
Spanish		Ac66	Rezende	Ac95	Ac0306
Spanish05	Redonda Red Spanish	Ac67	CB 65	Ac96	Ac0308
Spanish06	Red Spanish Pina Lisa	Ac68	CB 67	Ac97	Ac0401
Spanish07	Cabezona	Ac69	58-696		

Table 2. Summary of plant material used in AFLP analysis.

Species	Cultivar group	Number of plants
Plant material:		
A. ananassoides (Baker) L.B. Smith		4
A. bracteatus (Lindley) Schultes f.		7
A. comosus (L.) Merr.		(145)
	Cayenne	32
	Spanish	7
	Queen	5
	PRI	83
	Other	18
A. erectifolius L.B. Smith		2
A. nanus (L.B. Smith) L.B. Smith		1
Interspecific Hybrid		3
Total		162

The powdered samples were stored at −20 °C until DNA extractions were performed.

DNA extraction

DNA extraction of the *Ananas* samples was done according to Chittenden et al. (1994) with minor modifications. Approximately 7 mL of the previously lyophylized and powdered leaf sample was mixed with 20 mL extraction buffer (100 mM Tris, pH 8.0; 50 mM EDTA, pH 8.0; 500 mM NaCl; 2% polyvinylpolypyrrolidone; and 0.083% $NaHSO_3$) and incubated at 65 °C for 1 h with occasional shaking. Six-mL of 5 M potassium acetate was then added, and the tubes were mixed thoroughly by gentle inversion, placed on ice for 20 min, and centrifuged (Beckman Coultier, Allegra^{TM} 6R) at 3500 rpm for 20 min at 4 °C. The supernatant was filtered through one layer of Miracloth directly into 15 mL ice-cold (−20 °C) isopropanol, and incubated at −20 °C for 2 h. Precipitated DNA was pelleted by centrifugation at 3500 rpm for 20 min at 4 °C, and supernatant decanted. The pellet was washed once with purifying buffer (70% ethanol; 0.3 M sodium acetate), and once with 70% ethanol. The supernatant was removed, and the pellet was air-dried and resuspended in TE (10 mM Tris, pH 8.0; 1 mM EDTA, pH8.0). DNA samples were then treated once with phenol : chloroform : isoamyl alcohol (25 : 24 : 1) and centrifuged at 12 000 rpm for 15 min; the upper phase was collected, treated two times with chloroform: isoamyl alcohol (24 : 1), precipitated with 0.1 volume 3 M sodium acetate, and 2.2 volumes 95% ethanol. The DNA was then washed with 70% ethanol, air-dried, and resuspended in TE.

AFLP analysis

Genomic DNA digestion

AFLP analysis was performed according to Vos et al. (1995) with modifications as described by Steiger et al. (2002).

Preamplification and selective amplification

Preamplification and selective amplification conditions for the primer screen were done according to the procedures of Vos et al. (1995). The selective amplification primer combinations were chosen

Table 3. Numbers of AFLP markers generated from seven pairs of primers for 162 pineapple accessions and related species.

		Polymorphic markers	
*Eco*R I primer	*Mse* I primer	Set 1	Set 2
E-AAG	M-CAT	15	10
E-ACA	M-CTG	41	33
E-ACG	M-CAT	19	46
E-ACG	M-CTC	16	13
E-ACG	M-CTG	26	26
E-ACT	M-CAT	58	36
E-AGT	M-CTC	25	13
Total		200	177

during a screen of 72 primer sets on three accessions (Table 3). The initial primer survey was done with radioisotope γ^{32}P dATP labeling of the E-primers and fractionation of the AFLP selective amplification products on a 5% denaturing polyacrylamide gel.

AFLP analysis for 162 *Ananas* accessions was done using the Li-Cor IR^2 Automated DNA Sequencer (Li-Cor; Lincoln, NE). Upon selection of primer combinations to be used, IRDye^{TM} 700 and IRDye^{TM} 800 labeled *Eco*R I primers (Li-Cor, Lincoln, NE), and *Mse* I primers (Operon Technologies, Alameda, CA), were used in selective amplification, as described by Kim et al. (2002).

Data analysis

Two gels were run per primer set. Each gel image was scored independently because of the lack of consistent monomorphic (reference) bands shared by both gels, generating two data sets (Table 1). The gel images were analyzed utilizing the AFLP-Quantar^{TM} (v 1.0, KeyGene Product, Netherlands) analysis system. Bands were scored as present (+), absent (−), or unknown (?), first by the AFLP-Quantar^{TM} system, then refinements in scoring were made manually.

Scoring of the AFLP markers was difficult because of the numerous samples, high level of polymorphism, and conflicts with the gel analysis program. The high level of variation, and minimal monomorphic bands did not allow the two gels to be properly aligned; the gels were therefore scored separately. Scoring the gels separately minimizes the error that could be generated by incorrectly scoring AFLP products. Both data sets were run with seven primer combinations (Table 3). The data were scored in spreadsheet form and formatted for use with NTSYSpc (v. 2.1) cluster analysis software (Exeter Software, Co., NY). Pair-wise simple matching coefficients (Sokal and Michener 1958) were generated. Cluster analysis was performed with the unweighted pair group method using arithmetic means (UPGMA) algorithm (Sneath and Sokal 1973). The goodness of fit between the similarity and cophenetic matricies was evaluated by cophenetic correlation coefficient.

Results

The initial screen for polymorphic markers was carried out on three Smooth Cayenne cultivars, F153, F200, and D10. Among the 72 primer pairs surveyed, the average number of polymorphic markers was 23 with a range of 5–58. The seven most polymorphic primer sets were selected to genotype the entire pineapple germplasm collection. In total 200 AFLP markers were generated from the seven primer pairs for 83 *Ananas* samples in the first set and 177 markers for 79 samples in the second set (Table 3).

To verify that the markers being assessed were cultivar specific and not due to clonal variation, five individual plants from three cultivars, F153, F200, and D10, were examined with 16 primer sets. Virtually, no clonal variation was detected among individuals within each cultivar, except one of the five D10 plants had three extra bands from primer pair E-ACA/M-CAC and one extra band from primer pair E-ACA/M-CAT (data not shown).

Genetic similarity within established cultivar groups was estimated using pair-wise comparison of genetic similarity. The average genetic similarities of Cayenne were 0.761 and 0.810 for the Set 1 and Set 2 data, respectively; and ranged from 0.608 to 0.972 for Set 1, and 0.686 to 0.936 for Set 2. The most closely related Cayenne cultivars were F153 and F200, with an average genetic similarity of 0.972, whereas the least related cultivars were Cayenne Lot520WBC and Cayenne 7898QC, with an average genetic similarity of 0.608. The average genetic similarities of Spanish cultivars were 0.738 and 0.775 for the Set 1 and Set 2 data, respectively, and ranged from 0.676 to 0.800 (Set 1), and 0.743

to 0.827 (Set 2). The average genetic similarity of Queen cultivars was 0.726 for the Set 1 data, and ranged from 0.632 to 0.930 (only one Queen sample was included in Set 2).

Within *Ananas* species having multiple samples, the average genetic similarities for the two sets of *A. comosus* was 0.735. The average genetic similarity for the first set of 83 samples was 0.728 (ranging from 0.549 to 0.972), and 0.742 (ranging from 0.552 to 0.959) for the second set of 79 samples. A similar level of genetic similarity was observed in *A. ananassoides*, with average genetic similarity of 0.765. *A. bracteatus* appeared to be relatively less variable with an average genetic similarity of 0.877. With only two samples examined, the average genetic similarity of *A. erectifolius* L.B. Smith was 0.938. The average genetic similarities among *Ananas* species showed that *A. comosus* is most closely related to *A. ananassoides*.

Cluster analysis based on AFLP markers resulted in clear separation of each cultivar and accession, including all Smooth Cayenne cultivars (Figures 1 and 2). In the dendrogram generated from the first data set, the different *Ananas* species were divided into three major clusters (Figure 1). The first major cluster included all *A. comosus* samples. The second cluster consisted of the two *A. erectifolius* samples, four *A. ananassoides* samples, a hybrid derived from *A. comosus* and *A. ananassoides*, one *A. comosus*, and one *A. nanus* (L.B. Smith) L.B. Smith sample. The third cluster included only *A. bracteatus* samples. The cluster of *A. comosus* was further divided into four subclusters with no clear separation of major cultivar groups of Cayenne, Queen, and Spanish. The close genetic relatedness between *A. ananassoides* and *A. nanus* was demonstrated by the greater genetic similarity between these two species that was produced in the second major cluster. *A. ananassoides* and *A. erectifolius* clustered closely however, the average genetic similarity within each species (0.765 for *A. ananassoides* and 0.938 for *A. erectifolius*) was much greater than the average genetic similarity between these two species at 0.734.

The second dendrogram included only commercial cultivars and breeding lines of *A. comosus* along with three reference samples, one Cayenne cultivar F153, and one accession each from *A. ananassoidies* and *A. bracteatus* as outgroups (Figure 2). Seventy-nine samples of *A. comosus* were grouped into seven clusters. The first cluster included mostly Smooth Cayenne cultivars and the hybrids derived from these parental types (Table 1). There were no identical Cayenne cultivars based on their DNA fingerprinting pattern. Commercial cultivars and breeding materials received from private growers were dispersed among six of the seven clusters, reflecting the greater variation introduced by the breeding program of Hawaii's former Pineapple Research Institute. Queen and Spanish cultivars were likewise dispersed and did not form their own clusters.

Discussion

The ability to distinguish pineapple cultivars reliably could be invaluable for cultivar protection. The Cayenne cultivars are known to be derived from a few ancestral pineapple plants that originated from Cayenne, French Guiana (Collins 1960; Noyer 1991). These cultivars are thought to be a set of clones having little genetic variation. The narrow genetic stock and highly adaptive morphological characteristics have made cultivar distinction difficult. Vegetative reproduction and tissue culture propagation of these plants generates many phenotypic variant forms (Kaeppler et al. 2000; Cervera et al. 1998; Ray and Bingham 1990; Wakasa 1979; Collins 1960). Collins (1960) observed a high somatic mutation rate for some morphological traits such as spiny leaves and documented over 40 different types of mutations observed on leaves, flowers, and fruit, as well as non-morphological traits such as acidity and sugar content of fruit. These somatic mutations are the major source of variation used in the selection of new cultivars. These mutations may account for a small portion of the differences observed from DNA fingerprinting patterns. However, the average of more than 20% DNA marker differences among Cayenne cultivars suggests that a larger portion of the pineapple genome might have undergone changes than implied by the visible mutant phenotypes. Previous molecular studies have reported a low rate of genetic diversity within *A. comosus* but did not explore the diversity within a cultivar group (DeWald et al. 1988, 1992; Duval et al. 2001), mainly due to the limited number of isozyme and RFLP markers used. This is in

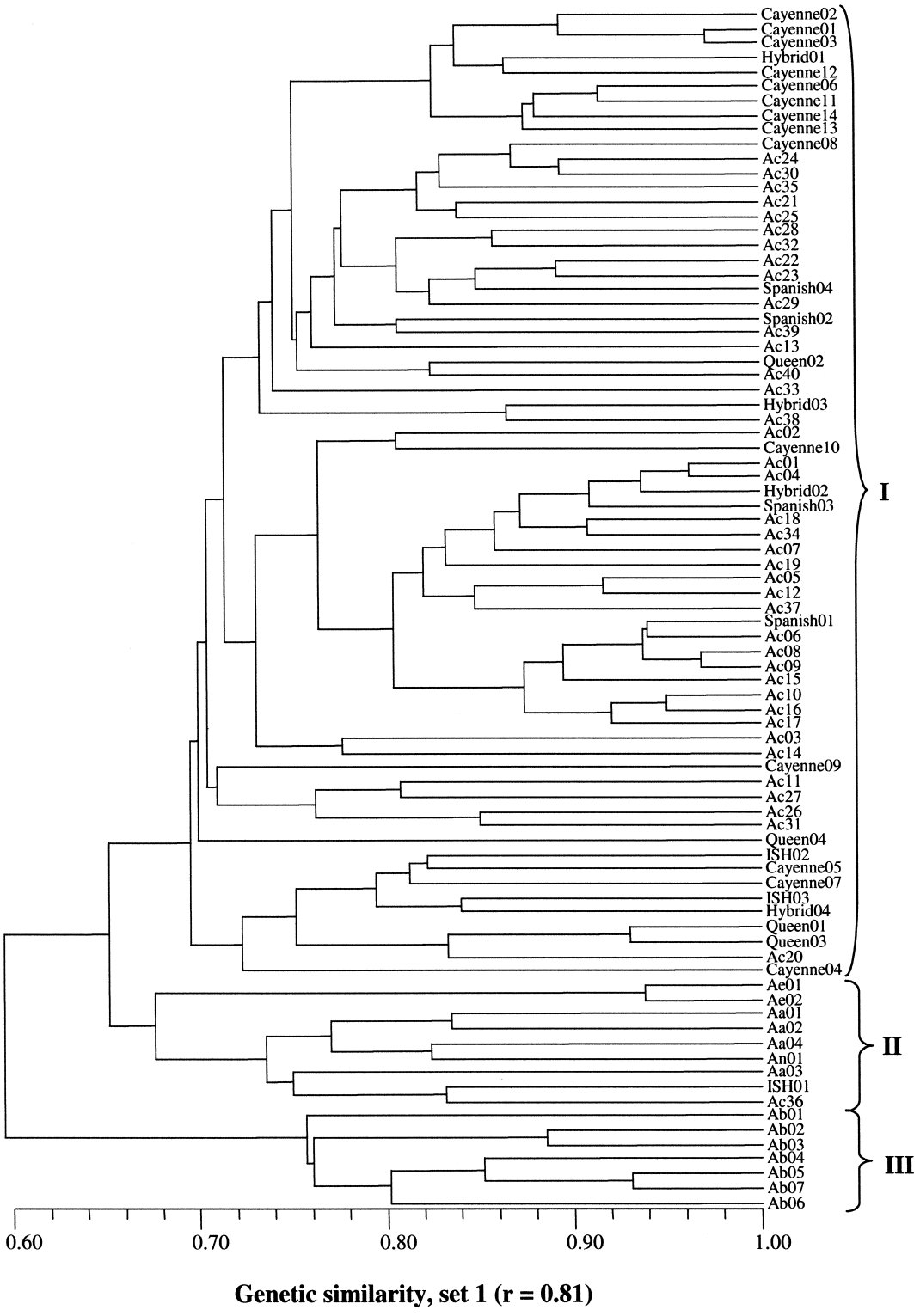

Figure 1. Cluster analysis of 83 accessions from five species of *Ananas*, including: *A. ananassoides* (Aa), *A. bracteatus* (Ab), *A. comosus* (Ac), *A. erectifolius* (Ae), and *A. nanus* (An), Intraspecific hybrid (Hybrid), and Interspecific hybrid (ISH).

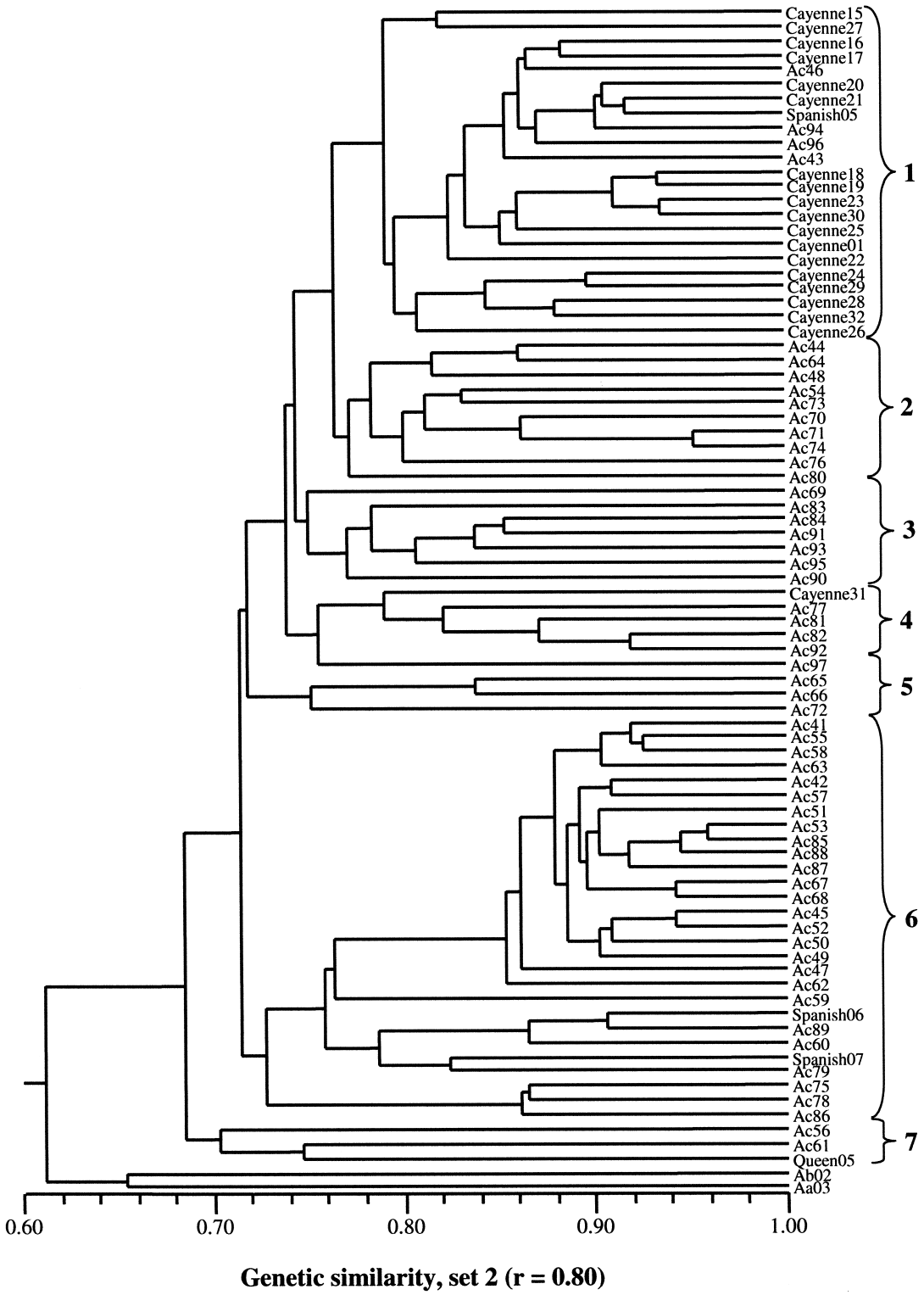

Figure 2. Cluster analysis of 79 accessions of *Ananas comosus*, and three reference samples: Cayenne cultivar F153 (Cayenne01), *A. ananassoides* (Aa), and *A. bracteatus* (Ab).

contrast to the present work showing the sensitivity of AFLP analysis that enables the identification of discrete DNA differences for each Cayenne cultivar.

Greater variations were observed among different types of cultivars. Genome instability, self-incompatability, and previously bred hybrids may be responsible for such variations. Genome rearrangements due to transposition might be responsible for genome instability and phenotypic variation (Kaeppler et al. 2000; Wakasa 1979). This type of activity within the genome coupled with interspecific fertilization due to self-incompatibility might explain the high degree of variation observed within Smooth Cayenne genotypes through AFLP markers (Brewbaker and Gorrez 1967). Rearrangements of redundant DNA would not be identified through isozymes because changes in genome structure frequently do not occur in the specific gene regions being measured (Powell et al. 1996).

The existing classification of cultivar groups is based on similarity of morphological characters. Although morphological characteristics differentiate the individual pineapple cultivars, *A. comosus* cultivar groups could not be distinctively separated using these AFLP markers. The possible explanation for this inability is that similar mutations occurred on different genetic background when these cultivars were selected and classified to cultivar groups, as suggested by Duval et al. (2001) for lack of clear separation of *Ananas* species using RFLP markers. It is known that one or a few genes can sometimes significantly change plant morphology. For example, the spiny or smooth leaf margins are controlled by a single gene (Collins 1960) possibly with three alleles (Kinjo 1993), and this trait is the signature character for the cultivar group Smooth Cayenne.

Pineapple improvement programs have evolved from selection of field variants, hybridization of different cultivars and species, and back to selection of superior field variants. Hybridization proved to be a difficult approach, because of the high level of genome heterozygosity possibly coupled with genome instability. Nevertheless, several low-acid commercial cultivars were developed by the more than 50 years of breeding efforts at the former Pineapple Research Institute (Williams and Fleisch 1993). Our results prove that there is considerable genetic variation among existing germplasm and provide indirect evidence for a high level of genome instability in pineapple. Under these circumstances, selection of field variants might still be the most valid approach for cultivar improvement in pineapple.

For cultivar identification using AFLP markers, multiple samples of the target cultivar as well as reference cultivars are necessary to ensure the accurate delineation of these cultivars based on their AFLP fingerprints. Although hardly any clonal variation was detected within the three established cultivars D10, F153, and F200, such measures would reduce or eliminate errors including mislabeling of samples and occasional DNA sample contamination.

Although the genetic diversity of *Ananas* species was not the focus of this project, a small number of accessions was collected from three additional species to be used as outgroups. The genetic diversity and relationships of these species assessed by AFLP markers did not agree with the results from isozyme and RFLP data (Aradhya et al. 1994; Duval et al. 2001). The genetic similarity of the four different species (*A. ananassoides*, *A. bracteatus*, *A. comosus*, and *A. erectifolius*) describes the highest species diversity in *A. comosus* (0.728) and *A. ananassoides* (0.765). *A. bracteatus* and *A. erectifolius* diversity levels were 0.877 and 0.938, respectively. Previous RFLP work by Duval et al. (2001) expressed diversity levels as number of bands/number of accessions and described *A. ananassoides* (100/56) as having the highest genetic diversity, followed by *A. bracteatus* (15/18) then *A. comosus* (62/167).

Ananas ananassoides holds a high level of genetic diversity. This species has been described as the wild pineapple species that is most widely distributed throughout South America. Its distribution has been documented throughout Brazil to northern Paraguay (Collins 1949). The wide distribution of this wild growing, sexually propagated species of *Ananas* reflects the high level of genome variation described in the molecular diversity studies above.

The RFLP study, unlike our AFLP analysis, showed that *A. bracteatus* has a higher degree of genetic diversity than *A. comosus* (Duval et al. 2001). The differences in the results may be due to the small sample number (7 accessions) of *A. bracteatus* tested by AFLP. Another explanation is that RFLP analysis assessed the polymorphisms

of 18 probes with low copy DNA sequences, while AFLP analysis described 200 (markers) differences of random genomic sequences. Additional samples will be needed to estimate the genetic variation within each species and to establish the genetic relationships among *Ananas* species.

This project started with the expectation that since the isozyme markers could not separate most smooth Cayenne accessions (Aradhya et al. 1994), we would identify a considerable degree of duplicated accessions among the germplasm maintained at the USDA, Tropical Plant Genetic Resource Management Unit at Hilo. However, our AFLP data provided sufficient resolution to identify the unique fingerprints for each accession. With hindsight, this is not surprising since isozyme markers detect the polymorphism of expressed genes, RFLP markers detect polymorphism of low copy DNA sequences, but AFLP markers detect polymorphism of both coding and non-coding genomic sequences. Therefore, both isozyme and RFLP markers are less polymorphic and less informative for distinguishing among accessions than AFLP markers based on the entire genome polymorphism.

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